IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Oreste, *et al.*
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Title: GLYCOSAMINOGLYCANS DERIVED FROM K5 POLYSACCHARIDE HAVING HIGH ANTICOAGULANT AND ANTITHROMBOTIC ACTIVITIES AND PROCESS FOR THEIR PREPARATION

Group Art Unit: 1623
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DECLARATION PURSUANT TO 37 C.F.R. §1.132

Sir:

We, Pasqua ORESTE, and Giorgio ZOPPETTI, do hereby declare as follows:

1. We are joint inventors of the instant application. Our Curriculum Vitaes are attached. We are familiar with the instant application and its prosecution as application number 09/950,003 and parent application number 09/738,879. We are also familiar with published pending application number 10/240,606, assigned to Inalco.
2. We have a financial interest in the instant application.
3. The invention of this application relates to

(3a) the observation that, when the final products (epik5-N,O sulfates) obtained at the end of step (g) are submitted to a nitrous depolymerization, the obtained depolymerized products (low molecular weight epik5-sulfates) normally lose or at least substantially lower their properties defined by the coagulation parameters; and

(3b) the discovery that (1) the observed loss of activity was at least partially due to the sulfation pattern of the partially O-desulfated intermediate obtained at the end of step(e) and (2) when carrying out the O-desulfation reaction step (e) by heating the oversulfated products for a period of time from 135 to 165 minutes selected from the known broad range of 1-8 hours, the nitrous depolymerization of the final epiK5-N,O-sulfate obtained after step (g) affords a depolymerized product which approaches the activity of the non-depolymerized epiK5-N,O-sulfate and has improved antithrombin activity, as defined by coagulation parameters. Compounds with unique sulfation patterns and activity, as defined in amended claims 38-53, were produced by these methods.

By consequence, this invention provides glycosaminoglycans having unexpected properties in respect of those of published application number 10/240,606, assigned to Inalco. These glycosaminoglycans were characterized by their ^{13}C-NMR spectra (Figures 15 and 16 of the parent application) and said spectra were interpreted in order to determine the percent of sulfo groups on the different hydroxyls of the glycosaminoglycans' chains. Said percent values are given in Examples 12 and 13 as well as in claims 11, 16 and 19 of the parent application 09/738,879.

4. After filing the parent application 09/738,879 and reviewing the ^{13}C-NMR spectra of Figures 15 and 16, we realized that we made a mistake in interpreting said spectra, so that we filed the present continuation-in-part application with the same Figures 15 and 16, properly interpreted. As a consequence, the last paragraphs of Examples 12 and 13 were replaced by new paragraphs with the correct percent values, while the activity data remained unchanged, and claims 11-26 of the parent application were replaced by claims 38-62 of instant application.
5. We, personally or under our direct supervision, carried out experiments in order to demonstrate the unexpected properties of the compounds of the present invention which are produced by the methods of this invention. In a first experiment, starting from an epiK5-N-sulfate obtained by epimerization with C5-epimerase from murine mastocytoma as in Examples 2 and 5, we carried out the solvolytic O-desulfation step (e) of the process (a)-(g), followed by a nitrous depolymerization, by heating a solution of the substrate in a dimethyl sulfoxide/methanol 9/1 (V/V) mixture at 60°C for 2, 2.5 and 3 hours. In a second experiment, starting from an epiK5-N-sulfate obtained by epimerization with immobilized recombinant C5-epimerase as described in Example 17, we carried out the solvolytic O-desulfation step (e) of the process (a)-(f), followed by a nitrous depolymerization, by heating a solution of the substrate in a dimethyl sulfoxide/methanol 9/1 (V/V) mixture at 60°C for 2.5, 3, 3.5 and 4 hours. The physico-chemical characteristics of the epiK5-N,O-sulfates obtained in the first and second experiments are summarized in tables 1 and 3, respectively.
6. The samples of the non-depolymerized and of the depolymerized epiK5-N,O-sulfates of each series of experiments were submitted to biochemical tests in order to determine the anti-Xa, anti-IIa, APTT and HCII activities. These biochemical tests were made under our direct supervision . The coagulation parameters of the epiK5-N,O-sulfates obtained in the first and second experiments are summarized in tables 2 and 4, respectively.
7. The results of the first experiment showed (a) that the depolymerized products deriving from the partially O-desulfated product treated at 2 and 3 hours actually underwent a substantial loss of activity on all the coagulation parameters, (that on the antithrombin (Anti-IIa) activity

being particularly severe), while the depolymerized products deriving from the partially O-desulfated product treated at 2.5 hours maintained a good activity level on all the coagulation parameters, (that on the antithrombin (anti-IIa) activity being particularly high, i.e. about three times that of heparin).

8. The results of the second experiment are consistent. This showed that the depolymerized products deriving from the partially O-desulfated product treated at 3, 3.5 and 4 hours underwent a loss of activity on all the coagulation parameters, while the depolymerized products deriving from the partially O-desulfated product treated at 2.5 hours maintained a good activity level on all the coagulation parameters, (that on the antithrombin (anti-IIa) activity being at least of the same order of magnitude as that of the non-depolymerized parent product and very high *per se*, i.e. about four times as high as that of heparin)
9. The difference in activity level illustrated in experiments 1 and 2 are significant and unexpected, e.g., a corresponding product with better activity could be used in lower amounts or could achieve a better effect at a given amount. These results are unexpected in that nothing in the prior art or in or experience led us to expect the established effect.
10. We declare further that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that the making of willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the applications or any patent issuing thereon.

Respectfully submitted,

Dated: 07/06/06

Pasqua ORESTE

Giorgio ZOPPETTI

EXPERIMENTAL PART

First experiment

Chemistry

I. Preparation of the epiK5-amine-O-oversulfate substrate (Preparation I)

(a) An amount of 10 g of polysaccharide obtained by fermentation as described in the Italian patent application MI99A001465 (WO 01/02597) with a purity of 80% (Fig.2 of instant application) was dissolved in deionized water to obtain a 1% solution. Triton X-100 was added to reach a concentration of 5% and the solution was kept at 55°C for 2 hours under stirring. The solution was brought to 75°C and kept at this temperature till a homogeneous turbid system is obtained and then the solution was rapidly cooled to room temperature. During the cooling two phases formed. Said thermal treatment was repeated twice on the upper phase (organic phase). The aqueous phase containing K5 polysaccharide was finally concentrated to 1/10 of its initial volume under reduced pressure and precipitated with acetone. The organic phase was discarded.

The product obtained is K5 polysaccharide with 90% purity detected by proton NMR (Fig. 3 of instant application).

(b) The product obtained in step (a) was dissolved in 1,000 ml of 2 N sodium hydroxide and kept at 60°C for 18 hours. The solution was cooled to room temperature and then brought to neutral pH with 6N hydrochloric acid. N-deacetylated K5 polysaccharide was obtained. The solution containing the N-deacetylated K5 was kept at 40°C and added with 10 g sodium carbonate in one step and 10 g of pyridine.sulfur trioxide adduct in 10 minutes. At the end of the reaction the solution was cooled to room temperature and then brought to pH 7.5-8 with a 5% hydrochloric acid solution. The obtained N-deacetylated N-sulfated K5 polysaccharide was purified from salts by diafiltration using a 1,000 D cut off spiral membrane (prepscale cartridge – Millipore). The purification process was stopped when the conductivity of the permeate was less than 100 μS. The product retained by the membrane was concentrated to 10% polysaccharide using the same diafiltration system and then was freeze dried to give K5-N-sulfate. The ratio N-sulfate/N-acetyl, measured at the ^{13}C-NMR spectrum was 9.5/0.5 (Fig 4 of instant application).

(c) An amount of 10 g of the K5-N-sulfate was dissolved in 1,000 ml of 25mM Hepes buffer pH 7.4 containing 40 mM $BaCl_2$. To this solution 1.5 x 10^{11}cpm of enzyme C5 epimerase extracted from murine mastocytoma as described by Jacobsson et al., J.Biol.Chem. 254 2975-2982 (1979), were added. The solution was kept at 37°C for 18 hours. The solution was then treated at 100°C for 10 minutes to denaturate the enzyme and finally was filtered on a 0.45 μ filter to obtain a clear solution containing the product. The product obtained was then purified by diafiltration and

precipitation with acetone. The pellet was dissolved in water at 10% concentration and submitted to step (d).

The epiK5-N-sulfate thus obtained showed a ratio iduronic acid/glucuronic acid 40.1/59.9.

(d) The 10% solution of epiK5-N-sulfate of step (c) was cooled to 10°C and then poured onto an IR 120 H^+ cationic exchange resin (50 ml). Both the column and the container of the eluted solution were kept at 10°C. After the passage of the solution the resin was washed with 3 volumes of deionised water. The pH of the flow through was more than 6. The acidic solution was brought to neutrality with an aqueous solution of 15% tetrabutylammoniun hydroxide. The solution was concentrated to 1/10 of its volume in a rotating evaporator under vacuum and freeze dried. The obtained tetrabutylammonium salt of epiK5-N-sulfate was suspended in 200 ml of DMF and added with 150 g of the adduct pyridine.SO_3 dissolved in 200 ml of DMF. The solution was kept at 45°C for 18 hours. At the end of the reaction the solution was cooled to room temperature and added with 1,200 ml of acetone saturated with sodium chloride. The obtained pellet was separated from the solvent by filtration, dissolved with 100 ml of deionised water and sodium chloride was added to 0.2 M concentration. The solution was brought to pH 7.5-8 with 2N sodium hydroxide and 300 ml of acetone were added. The pellet was separated by filtration. The obtained solid was dissolved in 100 ml deionised water and purified from the residual salts by diafiltration as described in step (b) to obtain Solution I.

The ^{13}C-NMR spectrum on a dried small amount of the obtained oversulfated product isolated from Solution I is shown in Fig. A. This spectrum is practically identical to that of the product of example 1(d) of instant application.

It results from said spectrum that said oversulfated glycosaminoglycan product presents the free amino group on glucosamine and O-sulfated groups on both glucosamine and iduronic/glucuronic units, for a sulfation degree of 3.2, i.e. it is an epiK5-amine-O-oversulfate (abbreviated as osGAG).

II. Preparation of three samples of epiK5-N,O-sulfates (Preparation II)

Solution I obtained at the end of Preparation I, containing the epiK5-amine-O-oversulfate substrate was submitted to steps (e)-(g), i.e. to solvolytic partial O-desulfation, 6-O-sulfation of the partially desulfated product thus obtained, N-sulfation of the 6-O-resulfated product according to the following protocol.

(e) Solution I containing epiK5-amine-O-oversulfate obtained at the end of Preparation I was passed onto a IR 120 H^+ cationic exchange resin. After the passage of the solution the resin was washed with 3 volumes of deionised water. The acidic solution was brought to neutrality with pyridine, then concentrated to 1/10 of its volume in a rotating evaporator at 40°C under vacuum and freeze dried. The obtained pyridine salt of the epiK5-amine-O-oversulfate was added with 1500 ml

of a solution of DMSO/methanol (9/1 V/V). The solution thus obtained was kept at 60°C and 50 ml aliquots were collected at the following times:

- after 2 hours in the case of Sample 1;
- after 2 hours and a half in the case of Sample 2;
- after 3 hours in the case of Sample 3,

and put in 50 ml of water to stop the reaction. The three samples were finally treated with 1,650 ml acetone saturated with sodium chloride. The solid obtained was purified by diafiltration as described in step (b) of Preparation I to obtain a solution at 10% concentration of a partially O-desulfated epiK5-amine-O-oversulfate. ^{13}C-NMR spectra of sample 1, 2 and 3 are reported in Fig. 1, 2 and 3 respectively. These spectra are informative of the sulfation pattern of the final epi K5-N,O sulfate, considering that almost complete 6-O and N-sulfation is obtained in steps (f) and (g).

(f) A volume of 50 ml of each of the three 10% solution samples of the partially O-desulfated epiK5-amine-O-oversulfate obtained at the end of step (e) was submitted to a 6-O-sulfation according to the following protocol.

A volume of 50 ml of the solution containing the product of step (e) was passed onto an IR 120 H^+ cationic exchange resin. After the passage of the solution the resin was washed with 3 volumes of deionised water. The acidic solution was brought to neutrality with a 15% of an aqueous solution of tetrabutylammoniun hydroxide. The solution was concentrated to 1/10 of its volume in a rotating evaporator under vacuum and freeze dried. The tetrabutylammonium salt of partially O-desulfated epiK5-amine-O-oversulfate thus obtained was suspended in 200 ml DMF. The suspension was cooled to 0°C and treated, in one step, with 40 g of pyridine.SO_3 adduct dissolved in 100 ml DMF. The solution was kept at 0°C for 1.5 hours and then treated with 750 ml acetone saturated with sodium chloride. The solid obtained was purified by diafiltration as described in step (b) of Preparation I to give a solution containing the 6-O-resulfated epiK5-amine-O-sulfate at a 10% concentration.

(g) A 50 ml sample of each of the three solutions obtained at the end of step (f) was submitted to N-sulfation exactly as described in step (b) of the Preparation I. Thus,

Sample 1A: an epiK5-N,O-sulfate obtained by carrying out the solvolytic, partial O-desulfation of step (e) in 2 hours;

Sample 2A: an epiK5-N,O-sulfate obtained by carrying out the solvolytic, partial O-desulfation of step (e) in 2 hours and a half;

Sample 3A: an epiK5-N,O-sulfate obtained by carrying out the solvolytic, partial O-desulfation of step (e) in 3 hours;

were isolated.

The ^{13}C-NMR spectrum of sample 2A is reported in Fig. 2A. 6-O sulfation and N-sulfation are evident from signals at 66-68 ppm and 58-60 ppm,

Each of the three samples of the final epiK5-N,O-sulfate obtained at the end of step (g) of Preparation II was submitted to nitrous depolymerization according to the protocol which follows.

III. Nitrous depolymerization of Samples 1A-3A (Preparation III)

An amount of 5 g of each sample was dissolved in 250 ml of water and cooled to 4°C with a thermostatic bath. The pH was brought to 2 with 1N hydrochloric acid cooled to 4°C and then 10 ml of a solution of 1% sodium nitrite were added. The pH was maintained to a value of 2 with 1N hydrochloric acid, the solution was kept under slow stirring for 15 minutes, then is neutralized with 1N NaOH, cooled to 4°C and then treated with a solution of 250 mg of sodium borohydride in 13 ml of deionised water. The reaction mixture was let to stand for 4 hours, then the pH was brought to 5 with 1N hydrochloric acid and, after 10 minutes neutralized with 1N NaOH. The depolymerized epiK5-N,O-sulfate thus obtained was recovered by precipitation with 3 volumes of ethanol and dried in a vacuum oven.

Sample 1B: Depolymerized epiK5-N,O-sulfate obtained from Sample 1A

Sample 2B: Depolymerized epiK5-N,O-sulfate obtained from Sample 2A

Sample 3B: Depolymerized epiK5-N,O-sulfate obtained from Sample 3A

The physico-chemical characteristics of Samples 1A-3A and 1B-3B are summarized in Table 1.

Table 1

Physico-chemical characteristics of epiK5-N,O-sulfates (epimerized using C5 epimerase extracted from murine mastocytoma, in solution), prepared according to Experiment 1.

Sample Final GAG	Step (e) Hours	MW (Da)	s.d.	Iduronic Acid %	Glucosam. N-S %	Glucosam. 6-OS %	Glucosam. 3-OS %	Id. Acid 2-OS %	Gl. Acid 3-OS %	Gl. Ac. 2-OS + Id. Ac. 3-OS
1A	2	10,100	2.9	40.1	>90	90	23	20	40	0
1B	2	7,800	2.9	40.1	>90	90	23	20	40	0
2A	**2.5**	**13,900**	**2.54**	40.1	**>90**	**90**	**17**	**26**	**31**	**0**
2B	**2.5**	**7,200**	**2.55**	40.1	**>90**	**90**	**17**	**26**	**31**	**0**
3A	3	12,600	2.51	40.1	>90	90	15	20	35	0
3B	3	7,300	2.52	40.1	>90	90	15	20	35	0

Hemobiology

The activity of samples 1A-3A and 1B-3B on the coagulation parameters, in particular their Anti-Xa, APTT, Anti-IIa (antithrombin) and HCII activities were tested according to the following methods.

1) Anti-Xa activity: Thomas D.P. et al. Thrombosis and Haemostasis 45 214 (1981) against the 4th International Standard of Heparin.
2) aPTT activity: Andersson et al. Thrombosis Research 9 575 (1976) against the 4th International Standard of Heparin.

3) HCII activity: the test was performed mixing 20 µl of a solution of 0.05 PEU (Plasma Equivalent Unit) /ml of HCII (Stago) dissolved in water with 80 µl of a solution of the sample under examination at different concentrations and 50 µl of Thrombin (0.18 U/ml-Boheringer) in 0.02M tris buffer pH 7.4 containing 0.15 M NaCl and 0.1% PEG-6,000. The solution was incubated for 60 seconds at 37°C, then 50 µl of 1mM chromogenic substrate Spectrozyme (American Diagnostic) were added. The reaction was continuously recorded for 180 seconds with determinations every second at 405 nm using an automatic coagulometer ACL 7000 (Instrumentation Laboratory).

4) Anti-IIa activity: the test was performed mixing 30 µl of a solution containing 0.5 U/ml of ATIII (Chromogenix) dissolved in 0.1M tris buffer pH 7.4 with 30 µl of a solution of the sample under examination at different concentrations and 60 µl of thrombin (5.3 nKat (Units of Enzymatic Activity)/ml-Chromogenix) in 0.1 M tris buffer pH 7.4. The solution was incubated for 70 seconds at 37°C, then 60 µl of 0.5 mM chromogenic substrate S-2238 (Chromogenix) in water was added. The reaction is continuously recorded for 90 seconds with determinations every second at 405 nm using an automatic coagulometer ACL 7000 (Instrumentation Laboratory).

The obtained results, expressed as the percent of activity in respect of heparin given as 100%, are summarized in Table 2, wherein "osStarting Material" indicates the oversulfated product obtained at the end of Preparation I and "Final GAG" indicates the final non-depolymerized or depolymerized epiK5-N,O-sulfate GlycosAminoGlycans.

Table 2

Activity on the coagulation parameters of heparin and epiK5-N,O-sulfates (epimerized using C5 epimerase extracted from murine mastocytoma, in solution), prepared according to Experiment 1.

osStarting Material	**Sample Final GAG**	**Step (e) Hours**	**Anti-Xa %**	**APTT %**	**Anti-IIa %**	**HCII %**
-	Heparin	-	100	100	100	100
Sample 1	1A	2	147	80.6	375	182
Sample 2	**2A**	**2.5**	**166**	**88**	**495**	**348**
Sample 3	3A	3	120	64	215	250
Sample 1	1B	2	61.8	41.6	37	77
Sample 2	**2B**	**2.5**	**66**	**50**	**290**	**148**
Sample 3	3B	3	40	41	36	91

Second Experiment

Chemistry

IV. Preparation of the epiK5-amine-O-oversulfate substrate (Preparation IV)

An amount of 10 g of K5 polysaccharide obtained by fermentation as described in the Italian application MI99A001465 (WO 01/02597) with a purity of 80% (Fig.2) was dissolved in deionized water to obtain a 1% solution. Triton X-100 was added to reach a concentration of 5% and the solution was kept at 55°C for 2 hours under stirring. The solution was brought to 75°C and kept at this temperature till a homogeneous turbid system was obtained and then the solution was rapidly cooled to room temperature. During the cooling two phases formed. Said thermal treatment was repeated twice on the upper phase (organic phase). The aqueous phase containing K5 polysaccharide was finally concentrated to 1/10 under reduced pressure and precipitated with acetone. The organic phase was discarded. The obtained product was K5 polysaccharide with 90% purity detected by proton NMR (Fig. 3) compared to the spectrum of the working standard (Fig. 1) and a retention time of 9 minutes on the HPLC analysis using two columns (Bio Rad Bio-sil SEC 250).

The process proceeded according to the following steps:

(i) The thus purified K5 was dissolved in 1,000 ml of 2 N sodium hydroxide and kept at 60°C for 18 hours. The solution was cooled to room temperature and then brought to neutral pH with 6N hydrochloric acid to give N-deacetylated K5 polysaccharide. The solution containing the N-deacetylated K5 polysaccharide was kept at 40°C and added with 10 g sodium carbonate in one step and 20 g of pyridine.SO_3 adduct in 10 minutes. At the end of the reaction the solution was cooled to room temperature and then brought to pH 7.5-8 with a 5% hydrochloric acid solution. The obtained product, K5-N-sulfate, was purified from salts by diafiltration using a 1,000 D cut off spiral membrane (prepscale cartridge - Millipore). The purification process was stopped when the conductivity of the permeate was less than 100 μS. The product retained by the membrane was concentrated to 10% polysaccharide using the same diafiltration system and then was freeze dried. The ratio N-sulfate/N-acetyl in the obtained product was 9.5/0.5 measured by ^{13}C-NMR (Fig. 4).

(ii) 1 - Preparation of the immobilized C5 epimerase

To 5 mg of recombinant C5 epimerase obtained as described by Jin-Ping L. et al., "Characterization of D-glucuronosyl-C5 epimerase involved in the biosynthesis of heparin and heparan sulfate", Journal Biological Chemistry, (2001) vol. 276, 20069-20077, corresponding to 1.2 x 10^{11} cpm (counts per minutes), dissolved in 200 ml of 25 mM Hepes buffer pH 7.4 containing 0.1 M KCl, 0.1% Triton X-100 and 0.015 M ethylenediaminotetracetic acid (EDTA), 100 mg of K5-N-sulfate obtained as described in step (i) were added. The solution was diafiltrated with a 30,000D

membrane at 4°C till disappearance of the starting K5-N-sulfate in the permeate. To the solution rententated by the membrane the buffer was changed by diafiltration against 200 mM $NaHCO_3$ at pH 7 and, after concentration to 50 ml, 50 ml of CNBr activated Sepharose 4B resin were added and kept to react overnight at 4°C. At the end of the reaction the amount of residual enzyme in the supernatant was measured with the Quantigold method (Diversified Biotec) after centrifugation. The enzyme in the supernatant was absent, showing that by operating according to this method the enzyme had been 100% immobilized. To occupy the sites still available the resin was washed with 100 mM tris pH 8. To measure the activity of the immobilized enzyme an amount of immobilized enzyme theoretically corresponding to 1.2×10^7 cpm was loaded into a column. In the obtained column 1 mg of K5-N-sulfate obtained as described in step (b) dissolved in 25mM Hepes, 0.1M KCl, 0.015 M EDTA, 0.01% Triton X-100, pH 7.4 buffer was dissolved, recirculating it through said column at 37°C overnight at a flow rate of 0.5 ml/minute. After purification by DEAE chromatographic system and desalting on a Sephadex G-10 the sample was freeze dried and analyzed for its content in iduronic acid by proton NMR technique as described in WO 96/14425. The ratio iduronic acid/glucuronic acid was 30/70.

2 - Epimerization.

An amount of 10 g of the N-sulfate K5 was dissolved in 600 ml of 25mM Hepes buffer pH 7 containing 50 mM $CaCl_2$. The solution obtained was recirculated through a 50 ml column containing the resin with the immobilized enzyme. This reaction was performed at 30°C with a flow rate of 200 ml/hour for 24 hours. The obtained product was purified by ultrafiltration and precipitation with ethanol. The pellet was dissolved in water at 10% concentration. An epimerized product was obtained with a ratio iduronic acid/glucuronic acid 54/46 against a ratio 0/100 of the starting material. The percentage of epimerization was calculated by ^{1}H-NMR.

(iii) The solution containing the epimerized product obtained in step (ii) was cooled to 10°C with a cooling bath and then applied onto a IR 120 H^+ 50 ml cationic exchange resin. Both the column and the container of the eluted solution were kept at 10°C. After the passage of the solution the resin was washed with 3 volumes of deionized water. The pH of the flow through was more than 6. The acidic solution was brought to neutrality with a 15% aqueous solution of tetrabutylammoniun hydroxide. The solution was concentrated to 1/10 of the volume in a rotating evaporator under vacuum and freeze dried. The product was suspended in 200 ml of dimethylformamide (DMF) and added with 150 g of the pyridine.SO_3 adduct dissolved in 200 ml of DMF. The solution was kept at 45°C for 18 hours. At the end of the reaction the solution was cooled to room temperature and added with 1,200 ml of acetone saturated with sodium chloride. The pellet obtained was separated from the solvent by filtration, dissolved with 100 ml of deionized water and sodium chloride was

added to 0.2 M concentration. The solution was brought to pH 7.5-8 with 2 N sodium hydroxide and 300 ml of acetone were added. The pellet was separated by filtration. The obtained solid was solubilized with 100 ml deionized water and purified from the residual salts by diafiltration as described in step (i) to obtain Solution IV.

V. Preparation of four samples of epiK5-N,O-sulfates (Preparation V)

Solution IV obtained at the end of Preparation IV, containing the epiK5-amine-O-oversulfate substrate, was submitted to steps (iv)-(vi), i.e. to solvolytic partial O-desulfation, 6-O-sulfation of the partially desulfated product thus obtained, N-sulfation of the 6-O-resulfated product according to the following protocol.

(iv) Solution IV containing the product of step (iii) was passed onto a IR 120 H^+ cationic exchange resin. After the passage of the solution the resin was washed with 3 volumes of deionized water. The pH of the flow through was more than 6. The acidic solution was brought to neutrality with pyridine. The solution was concentrated to 1/10 of its volume in a rotating evaporator at 40°C under vacuum and freeze dried. The product obtained as pyridine salt was added with 1500 ml of a solution of DMSO/methanol (9/1 V/V). The solution thus obtained was kept at 60°C and 50 ml aliquots were collected at the following times:

- after 2 hours and a half in the case of Sample 4;
- after 3 hours in the case of Sample 5;
- after 3 hours and a half in the case of Sample 6;
- after 4 hours in the case of Sample 7,

then added to 50 ml deionized water and finally treated with 1,650 ml acetone saturated with sodium chloride. The solid obtained was purified by diafiltration as described in step (i) of Preparation IV to obtain a solution at 10% concentration of a partially O-desulfated epiK5-amine-O-oversulfate.

The ^{13}C-NMR spectra of samples 4, 5, 6 and 7 are reported in Fig. 4, 5, 6 and 7 respectively.

(v) A volume of 50 ml of each of the four 10% solution samples of the partially O-desulfated epiK5-amine-O-oversulfate obtained at the end of step (iv) was submitted to a 6-O-sulfation according to the following protocol.

A volume of 50 ml of the solution containing the product of step (iv) was passed onto a IR 120 H^+ cationic exchange resin (50 ml). After the passage of the solution the resin was washed with 3 volumes of deionized water. The pH of the flow through was more than 6. The acidic solution was brought to neutrality with an aqueous solution of 15% tetrabutylammoniun hydroxide. The solution was concentrated to 1/10 of the volume in a rotating evaporator under vacuum and freeze dried. The product as tetrabutylammonium salt was suspended in 200 ml DMF. The suspension was cooled to

0°C and treated with 40 g of the pyridine.SO_3 adduct dissolved in 100 ml DMF. The sulfating agent was added one step. The solution was kept at 0°C for 1.5 hours and then treated with 750 ml acetone saturated with sodium chloride.

The solid obtained was purified by diafiltration as described in step (i) of Preparation IV to give a solution containing the 6-O-resulfated epiK5-amine-O-sulfate at a 10% concentration.

(vi) A 50 ml sample of each of the four solutions obtained at the end of step (v) was submitted to N-sulfation exactly as described in step (i) of Preparation IV. Thus,

Sample 4A: an epiK5-N,O-sulfate obtained by carrying out the solvolytic, partial O-desulfation of step (iv) in 2 hours and a half;

Sample 5A: an epiK5-N,O-sulfate obtained by carrying out the solvolytic, partial O-desulfation of step (iv) in 3 hours;

Sample 6A: an epiK5-N,O-sulfate obtained by carrying out the solvolytic, partial O-desulfation of step (iv) in 3 hours and a half

Sample 7A: an epiK5-N,O-sulfate obtained by carrying out the solvolytic, partial O-desulfation of step (iv) in 4 hours;

were isolated.

VI. Nitrous depolymerization of Samples 4A-7A (Preparation VI)

Each of the four samples obtained at the end of step (vi) of Preparation V was submitted to nitrous depolymerization according to a protocol identical with that of Preparation III. Thus,

Sample 4B: a depolymerized epiK5-N,O-sulfate obtained by carrying out the solvolytic, partial O-desulfation of step (iv) in 2 hours and a half;

Sample 5B: a depolymerized epiK5-N,O-sulfate obtained by carrying out the solvolytic, partial O-desulfation of step (iv) in 3 hours;

Sample 6B: a depolymerized epiK5-N,O-sulfate obtained by carrying out the solvolytic, partial O-desulfation of step (iv) in 3 hours and a half

Sample 7B: a depolymerized epiK5-N,O-sulfate obtained by carrying out the solvolytic, partial O-desulfation of step (iv) in 4 hours;

were isolated.

The ^{13}C-NMR spectrum of sample 4B is reported in Fig. 4B. Signals from anhydromannitol residues formed by depolimerization are shown from 80 and 85 ppm and from 60 and 62 ppm.

The physico-chemical characteristics of Samples 1A-3A,1B-3B, 4A-7A and 4B-7B are summarized in Table 3.

Table 3

Physico-chemical characteristics of epiK5-N,O-sulfates (epimerized using immobilized, recombinant C5 epimerase), prepared according to Experiment 2.

Sample Final GAG	Step (iv) Hours	MW (Da)	s.d.	Iduronic Acid %	Glucosam. N-S %	Glucosam. 6-OS %	Glucosam. 3-OS %	Id. Acid 2-OS %	Gl. Acid 3-OS %	Gl. Ac. 2-OS + Id. Ac. 3-OS
4A	**2.5**	**14,900**	**2.7**	**54**	**>90**	**90**	**20**	**30**	**35**	**5**
4B	**2.5**	**6,900**	**2.7**	**54**	**>90**	**90**	**20**	**30**	**35**	**5**
5A	3	14,910	2.25	54	>90	85	18	12	20	0
5B	3	7,500	2.25	54	>90	85	18	12	20	0
6A	3.5	14,750	2.43	54	>90	85	18	20	25	5
6B	3.5	7,200	2.43	54	>90	85	18	20	25	5
7A	4	15,540	2.41	54	>90	89	15	20	20	5
7B	4	8,000	2.41	54	>90	89	15	20	20	5

Hemobiology

The activity of Samples 4A-7A and 4B-7B on the coagulation parameters, in particular their Anti-Xa, APTT, Anti-IIa (antithrombin) and HCII activities were tested as described above for the first experiment.

The obtained results, expressed as the percent of activity in respect of heparin given as 100%, are summarized in Table 4.

Table 4

Activity on the coagulation parameters of heparin and epiK5-N,O-sulfates (epimerized using immobilized, recombinant C5 epimerase), prepared according to Experiment 2.

osStarting Material	Sample Final GAG	Step (iv) Hours	Anti-Xa %	APTT %	Anti-IIa %	HCII %
-	Heparin	-	100	100	100	100
Sample 4	**4A**	**2.5**	**166**	**76**	**400**	**283**
Sample 5	5A	3	150	67	386	292
Sample 6	6A	3.5	132	69	312	254
Sample 7	7A	4	167	76	334	300
Sample 4	**4B**	**2.5**	**99**	**74**	**416**	**134**
Sample 5	5B	3	83.4	64	304	121
Sample 6	6B	3.5	69.4	60	212	113
Sample 7	7B	4	77	61	248	103.5

Dated 07/06/06

Pasqua ORESTE

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